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Polymerase Chain Reaction Assay for Avian Polyomavirus

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A polymerase chain reaction assay was developed for detection of budgerigar fledgling disease virus (BFDV). The assay used a single set of primers complementary to sequences located in the putative coding region for the BFDV VP1 gene. The observed amplification product had the expected size of 550 bp and was confirmed to derive from BFDV DNA by its restriction digestion pattern. This assay was specific for BFDV and highly sensitive, being able to detect as few as 20 copies of the virus. By using the polymerase chain reaction, BFDV was detected in adult, nestling, and embryo budgerigar (*Melopsitticus undulatus*) tissue DNAs and in sera from adult and nestling budgerigars. These results suggest the possibility of persistent infections in adult birds and lend further support to previously described evidence of possible in ovo transmission. BFDV was also detected in chicken embryo fibroblast cell cultures and chicken eggs inoculated with the virus. A 550-bp product with identical restriction enzyme sites was amplified from a suspected polyomavirus isolated from a peach-faced lovebird (*Agapornis pesonata*) and from tissue DNA from a Hahn's macaw (*Ara nobilis*) and a sun conure (*Aratinga solstitialis*) with histological lesions suggestive of polyomavirus infection. These fragments also hybridized with a BFDV-derived probe, proving that they were derived from a polyomavirus very similar, if not identical, to BFDV.

The virus that causes the so-called budgerigar fledgling disease has been characterized (4, 5, 20), cloned (19), and sequenced (27). These results have demonstrated that it is a true polyomavirus and has many similarities to the mammalian polyomaviruses. Karyomegaly in cells of many organs, often with intranuclear inclusion bodies, is a common finding in birds with budgerigar fledgling disease virus (BFDV) infections (2, 3, 5, 13). These lesions have been described in nestlings as young as 1 day of age, raising the possibility of transmission of the virus in the egg (3). A disease producing similar histological lesions containing virus particles characteristic of the *Papovaviridae* has been described in several species of hand-fed nestling parrots (12, 16), 4- and 8-month old splendid parakeets (*Neophema splendida*) (23), adult lovebirds (*Agapornis* spp.) (22), adult finches (17, 31), and fledgling and immature Gouldian finches (*Erythrura gouldiae*) (10). Ultrastructural evaluation (12, 16), cross-reactivity with a fluorescent-antibody conjugate produced against BFDV, and cytopathic effects in chicken embryo fibroblasts (CEFs) (12) suggest that the virus(es) that causes the disease in hand-fed parrots is a polyomavirus which is closely related to BFDV.

A review of cases submitted to the diagnostic service of the Schubot Exotic Bird Health Center showed that of 2,026 cases submitted from January 1989 through May 1990, BFDV was identified in eight breeding operations, and the disease was diagnosed in 57 parrots other than budgerigars. These figures indicate that both of these diseases still threaten the aviculture industry in the United States.

In the past, diagnosis of BFDV or suspected avian polyomavirus infections has depended on finding characteristic lesions, positive fluorescent-antibody conjugate staining of impression smears or frozen sections, electron microscopic examination of affected organs, or virus isolation through egg inoculation or inoculation of cell cultures. Also, in order to study these viruses, virus isolation has been necessary. Virus isolation, however, is time-consuming, often requiring repeated passages through eggs or cell cultures, and has proved to be difficult. To date, there is only one report of a suspected polyomavirus isolated from psittacine species other than budgerigars (12).

Recently, the polymerase chain reaction (PCR), a method for the in vitro amplification of target DNA sequences, has been applied as a quick diagnostic tool for the detection of virus genome in clinical specimens, including urine (1, 21), sera (18), feces (11), and DNA extracted from fresh (1, 26, 32) and Formalin-fixed, paraffin-embedded tissues (14, 26, 30). In this report we describe the use of PCR to identify a BFDV sequence in budgerigar tissue DNA, serum, tissue culture fluid, and allantoic fluid. Using the same method, we also demonstrate that BFDV DNA sequences or closely related sequences can be identified in tissues from other parrot species with lesions characteristic of budgerigar fledgling disease.

MATERIALS AND METHODS

Specimen collection and preparation. Budgerigar (*Melopsittacus undulatus*) DNA and sera were obtained from embryos (estimated age range, 5 to 17 days of incubation), nestlings (weight, 11 to 21 g), and adult birds submitted for diagnostic necropsy from two breeding operations with a past history of BFDV-associated mortality. Individual birds were anesthetized by intramuscular injection of a 50:50 dilution of ketamine hydrochloride (Ketaset; 100 mg/ml; Aveco Corp., Fort Dodge, Iowa) and xylazine (Rompun; 2 mg/ml; Haver, Shawnee, Kans.). Each bird was exsanguinated by heart puncture, and its feathers were thoroughly wetted with a 0.5% solution of disinfectant (Roccal-D; Winthrop Veterinary, New York, N.Y.); the bird was then dissected with instruments which were washed, dried, soaked in alcohol, and flamed between necropsies. Portions of the heart, spleen, liver, and kidney were stored in a single

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container at −85°C until they were processed. Tissues were also collected for histologic examination. Each tissue was cut into sections less than 5-mm thick, fixed in 10% buffered Formalin, and embedded in paraffin. Six-micrometer sections of the paraffin-embedded tissues were stained with hematoxylin-eosin. The blood was allowed to clot; the serum was separated from the clot by centrifugation and stored at −85°C until it was processed. Two cases, a Hahn's macaw (*Ara nobilis*) and a sun conure (*Aratinga solstitialis*), were selected from necropsy submissions in which lesions suspected to be caused by a polyomavirus were observed. They were chosen because frozen liver and spleen and Formalin-fixed tissues were available from both birds. BFDV was isolated from birds collected from the two breeding colonies and amplified in CEF cell cultures as described by Purchase (24). A suspected polyomavirus previously isolated by Graham and Calnek (12a) from a peach-faced lovebird (*Agapornis roseicollis*) was propagated in CEF cell cultures and was inoculated onto chorioallantoic membranes (24). Control samples included DNA extracted from the liver of a peach-faced lovebird which died with the so-called feather and beak disease, from CEF cell cultures, and from peripheral blood cells collected from an apparently healthy yellow-collared macaw (*Ara auricollis*) and a Patagonian conure (*Cyanoliseus patagonus*). Cesium chloride-purified DNAs from human papillomavirus type 1 (HPV-1) (ATCC 45021), human papillomavirus type 6 (HPV-6), bovine papillomavirus type 1 (ATCC pdBPV-1 37134), deer papillomavirus, and simian virus 40 (SV40) were used as controls. One nanogram of each viral DNA was used for the PCR. Because all the papillomavirus DNAs were cloned in plasmids, 1 ng corresponded to approximately 10^8 viral genome copies. The SV40 sample was genomic viral DNA which corresponded to approximately 2×10^8 copies per ng.

DNA preparation. Tissue samples, which consisted of approximately 1 g of pooled organ tissue, 0.1 ml of clotted blood, or entire embryos, were ground in a hand-held tissue grinder in 2 ml of 0.1× TE (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.5])–1% sodium dodecyl sulfate. The suspensions were then digested with proteinase K for 12 to 18 h at 37°C. The solutions were extracted two to three times with phenol and once with chloroform. The aqueous phase was made to 0.3 M with respect to sodium acetate, and the nucleic acids were precipitated by the addition of 2.5 volumes of ethanol and storage at −20°C overnight. One gram each of pooled Formalin-fixed liver and spleen from both the Hahn's macaw and sun conure cases was minced and washed separately five times in 0.1× TE. DNA was then extracted in the same manner as the fresh tissues were. All DNA samples were resuspended in 0.1× TE, and their concentrations were estimated by absorption of UV light at a wavelength of 260 nm. One g of cellular DNA, approximately the equivalent of the DNA from 5×10^5 cells (7), in 1 to 3 μl of buffer was used for each PCR. Uninfected CEF cell cultures (2.4×10^6 cells per ml) and cell cultures infected with either BFDV or the lovebird viral isolate were frozen (−85°C) and thawed three times. One microliter of this suspension was added directly to the PCR mixture, or 150 μl of the suspension was boiled for 10 min. The boiled samples were centrifuged at 2,000 × *g* for 5 min, and 1 μl of the clear liquid between the pellet and the lipid layer from each sample was then added to the PCR mixture. Other cell suspensions were pelleted at $1.26 \times 10^5 \times g$ for 2 h. The pellets were resuspended in 0.1× TE and processed in the same manner as the tissue samples were. Allantoic fluid was collected and pooled from chicken eggs inoculated with the lovebird viral isolate and from chicken eggs that were sham inoculated with tissue culture medium. One microliter of unprocessed allantoic fluid was added to the reaction mixture.

TABLE 1. Optimized temperature and time parameters for PCR

Cycle	Melting phase (min/°C)	Annealing phase (min/°C)	Extension phase (min/°C)	Cycle repetitions (no.)
1	5/94	2/48	1/72	1
2	1/94	2/48	1/72	40
3	1/94	2/48	10/72	1

Selection of primers. Primer A (CTTATGTGGGAGGCTG CAGTGTT, positions 2183 to 2206) and primer B (TACTGA AATAGCGTGGTAGGCCTC, positions 2709 to 2732) were selected from two 60-bp segments (positions 2161 to 2220 and positions 2701 to 2760) so that they would flank a portion of the large open reading frame believed to code for the BFDV VP1 protein (27). This region was selected because it has been shown to be an area with relatively high sequence homology between polyomaviruses (27). Therefore, it was thought that these primers might amplify not only BFDV but also other closely related viruses. A computer program (PCR Primer Selection; Epicenter Software, Pasadena, Calif.) was used to select these primers based on minimal intra- and interprimer complementarity and the lack of a significant secondary structure. Although several sequences met these criteria, these primers were chosen because they were of equal length and had the same G+C content (50%). Primers were synthesized by Oligonucleotide Synthesis Services (College Station, Tex.) and purified by dissolving 0.1 pmol of each separately in 100 μl of 0.1× TE buffer and precipitating them in 2.5 volumes of isopropyl alcohol at −20°C overnight.

Amplification conditions. PCR was carried out in 500-μl polypropylene microfuge tubes with a total reaction volume of 100 μl. Various concentrations of $MgCl_2$, *Taq* polymerase, oligonucleotide primers, and deoxynucleotides were tested. An optimized reaction mixture containing 3 mM $MgCl_2$; 1 U of *Taq* polymerase; 0.2 μM (each) CTP, TTP, ATP, and GTP; 50 mM KCl; 1 mM Tris base; 0.001% gelatin; and 4 μg each of oligonucleotide primers A and B per ml was used for each of the clinical specimens. After the addition of the various clinical specimens, the reaction mixture was overlaid with 75 μl of reagent-grade light mineral oil. Thermocycling was carried out in a programmable heating block (Eppendorf Microcycler; Brinkmann Instruments, Freemont, Calif.), with cooling water maintained at 5°C by attachment to a circulating water bath. Annealing temperatures, extension times, and the number of repetitions of the amplification cycle were varied. A single optimal set of parameters was found and was used for the clinical samples (Table 1). Following the third cycle, the samples were immediately cooled to 6°C.

Detection of the amplified products. A 15-μl aliquot of the reaction mixture was electrophoresed for 45 min on an 80-ml, 1% agarose gel containing 10 μg of ethidium bromide. The tank buffer was 1× TBE (89 mM Tris base, 89 mM sodium borate, 2 mM EDTA [pH 8.0]). Gels were illuminated with UV light and photographed. For Southern blot analysis, the DNA was transferred from the gels to a nylon membrane (Nytran; Schleicher & Schuell Co., Keene, N.H.) by alkaline denaturation and the capillary method of Reed and Mann (25). The DNA probe was produced by isolating

the 550-bp product amplified from a single budgerigar and by using this as a template in a modification of the procedures described by Feinberg and Vogelstein (8, 9). Briefly, agar containing the visible 550-bp amplification product was cut into small blocks. The blocks were placed in wells of a DNA electroelutor (model UEA Unidirectional Electroelutor; IBI, New Haven, Conn.) with a tank buffer of 0.5× TBE, and the DNA was electroeluted into 3 M ammonium acetate. Ethidium bromide was extracted from the DNA solution with isoamyl alcohol, and the DNA was precipitated with ethanol and resuspended in 0.1× TE. Two hundred nanograms of the purified 550-bp product was denatured by boiling for 5 min, quenched on ice, and added to a total reaction mixture containing 10 μl of oligo-labeling buffer (1.0 M HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid]; 0.25 M Tris; 25 mM $MgCl_2$; 0.1 mM [each] dGTP, dCTP, and dTTP [pH 7.5]), 3 μl of each oligonucleotide primer (A and B) solution (0.133 mg/ml), 2 μl of 1% bovine serum albumin, 0.5 μl of the Klenow fragment of DNA polymerase I (0.5 U), and 5 μl of [^{32}P]dATP (20 μCi/μl). The reaction mixture was then incubated at 25°C overnight and stopped by the addition of three times its volume of a solution containing 0.1% sodium dodecyl sulfate and 2.5 mM EDTA. The probe was purified on a Sephadex G-50 column and used within 7 days of preparation. An initial specific activity of 3×10^8 cpm/μg of template DNA or higher was detected in each batch of probe used. Nylon membranes containing the transferred DNA were neutralized by soaking them with agitation in 200 ml of 2× SSPE (0.36 mM NaCl, 0.20 mM $NaPO_4$, 2 mM EDTA [pH 7.7]) for 30 min. Membranes were placed in a siliconized glass tube with 15 ml of prehybridization solution (1.5× SSPE, 0.5% BLOTTO [10% {wt/vol} nonfat dry milk], 100 μg of denatured herring sperm DNA per ml, 0.1% sodium dodecyl sulfate) and incubated for 6 to 12 h at 68°C in a hybridization incubator (Robbins Scientific Corp, Sunnyvale, Calif.). The prehybridization buffer was replaced with 7 ml of hybridization buffer (prehybridization buffer containing 10^6 cpm of viral probe per ml) and incubated for 12 to 18 h at 68°C. After hybridization, the membranes were washed under conditions of high stringency and were air dried. Autoradiography was carried out at −85°C. Exposure times were 2, 24, and 48 h.

Quantitation of amplified products. The sensitivity of the reaction was estimated by making 10-fold serial dilutions of a known concentration of viral DNA purified from the lovebird viral isolate. These dilutions were added directly to the PCR mixture or added with 1 μg of cell DNA extracted from a whole chicken embryo. To estimate the original viral copy number in the clinical samples, the amplified products from two dilutions of virus DNA (20 [0.1 fg] and 2×10^4 [100 fg] copies) amplified in the presence of 1 μg of chicken embryo cellular DNA were used as internal controls on each agarose gel. Positive samples were recorded on a scale of + to ++++ based on the brightness of the ethidium bromide-stained band or the intensity of the band produced on autoradiography. After a 48-h exposure period, samples with a faint but definite band equal to the product of 20 viral copies were considered as +. Samples darker than the product of 20 copies (0.1 fg) but not visible on the agarose gel were recorded as ++. Samples visible on the agarose gel and of equal or lesser intensity than the product of 2×10^4 copies (100 fg) were recorded as +++. Brighter samples were recorded as ++++.

Restriction enzyme digestion. Five units of each of four restriction enzymes, *Taq*I, *Bam*HI, *Alu*I, and *Pst*I, and 1 μl of their appropriate 10× buffers were added individually to 10 μl of the final PCR mixture containing amplified product from each of the four species of birds examined. Following a 45-min incubation period, the entire reaction mixture was loaded onto a 1.5% agarose gel containing 0.125 μg of ethidium bromide per ml of agar and electrophoresed for 45 min at 110 V. The bands were observed and photographed as described above.

Sequence analysis of other polyomaviruses. The sequences of SV40, JC virus, and the mouse polyomavirus were searched with a computer program (Super Clone; Coral Software, San Diego, Calif.) for sequences which would have homology with the selected primers.

RESULTS

Optimization of the restriction conditions. Precise reaction conditions were necessary for maximum sensitivity. $MgCl_2$ and *Taq* polymerase concentrations were especially critical. Previous observations have shown that the $MgCl_2$ concentration in the reaction mixture can affect both the sensitivity and the specificity of the PCR and that optimum concentrations vary widely for different oligonucleotide primers (15, 28). With the nucleotide primers used here, an optimum final reaction concentration of 3 mM $MgCl_2$ produced the best product yield. A slight increase or decrease (17%) in the $MgCl_2$ concentration resulted in a substantial reduction in yield (data not shown). One unit of *Taq* polymerase was found to be optimal, while an increase or decrease of as little as 0.5 U resulted in a substantial reduction in product (data not shown). Annealing temperatures can also affect the specificity of the primers; low annealing temperatures may permit primer hybridization with similar, but not identical, nucleotide sequences. With these primers 48°C was found to be optimal. A 5°C increase resulted in a substantial decrease in product, although there was no decrease in the nonspecific amplification products (data not shown). Extension and melting temperatures were not varied, but extension times of 1 and 3 min yielded no apparent differences in product amounts. Therefore, an extension time of 1 min was chosen, thereby reducing the total length of each run.

Determination of the sensitivity of the PCR. The sensitivity of the PCR was tested by using 10-fold dilutions of purified viral DNA in the absence (40 repetitions of cycle 2) or presence (30 and 40 repetitions of cycle 2) of 1 μg of cellular DNA. In the presence of cellular DNA, a minimum of 2×10^5 copies of viral genome was required to produce a visible band on the ethidium bromide-agarose gel. By increasing the cycle repetitions to 40, the amplified product from as few as 2×10^3 copies of virus was detected on the ethidium bromide-agarose gel (Fig. 1A). Cellular DNA interfered with the sensitivity, because 200 viral copies were detected after amplification with 40 repetitions if virus DNA was added alone (data not shown). Southern hybridization, using the ^{32}P-labeled probe derived from BFDV, further increased the sensitivity of the reaction. In the presence of cellular DNA, the product from as few as 2×10^3 viral copies was detected with 30 repetitions of cycle 2, while 40 repetitions produced a visible product from as few as 20 copies in the reaction mixture (Fig. 1B). Without cellular DNA, the product from as few as two viral copies resulted in a detectable band (data not shown). Because the cycle repetition was increased, more nonspecific amplification occurred which resulted in more background staining and brighter nonspecific bands.

Primer specificity. To determine the specificity of the nucleotide primers, DNA from tissue suspected of containing BFDV was assayed by PCR in parallel with non-BFDV

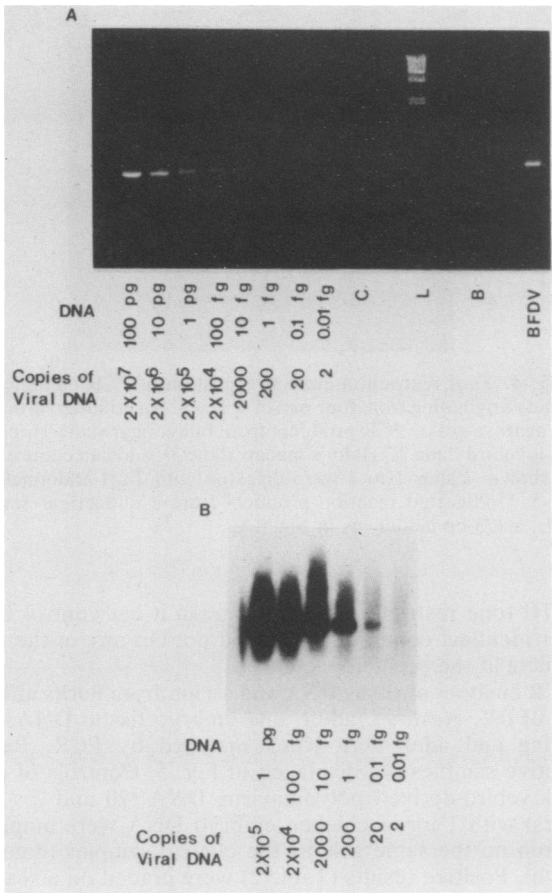

FIG. 1. Determination of the sensitivity of PCR. (A) Reaction mixtures containing 1 μg of chicken embryo DNA and serial 10-fold dilutions of avian polyomavirus DNA isolated and purified from a lovebird were amplified by PCR (40 repetitions of cycle 2). Fifteen microliters of each reaction mixture was electrophoresed on an ethidium bromide–1% agarose gel. The amount of viral DNA and the corresponding number of viral genome copies added to the initial reaction mixture are indicated beneath the lanes. Amplified product could be detected when the original reaction mixture contained as few as 2,000 copies (10 fg) of the virus. The lane marked BFDV contains the amplification products from a typical sample of nestling budgerigar tissue DNA (1 μg). A *Hin*dIII digest of lambda phage DNA (lane L) was included as a molecular mass marker. The lowest visible band represents a 564-bp fragment. Amplified products from 1 μg of chicken embryo DNA with no viral DNA added (lane C) and the reaction mixture with no DNA added (lane B) were included as controls. (B) The DNA fragments from the agarose gel were transferred to a nylon membrane and hybridized with a ^{32}P-labeled BFDV-derived probe. The hybridized filter was exposed to radiographic film for 48 h. The radiolabeled probe detected the amplification product from an original reaction mixture containing as few as 20 viral copies (0.1 fg).

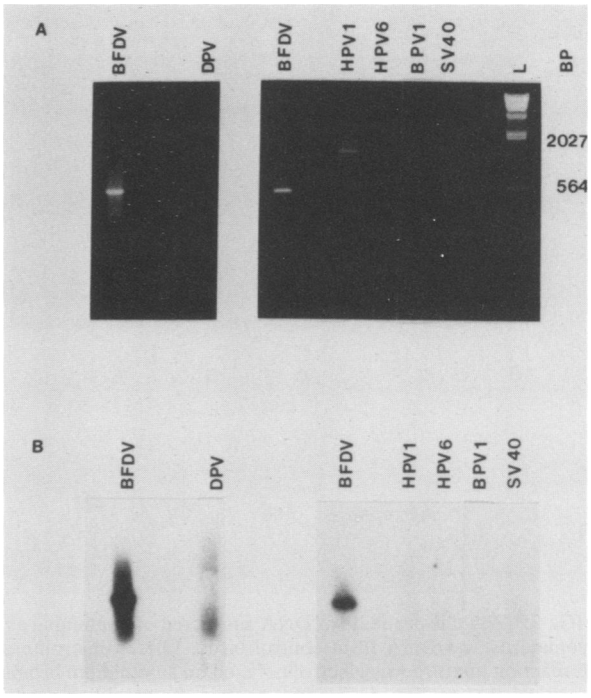

FIG. 2. (A) PCR-amplified products of BFDV and control viruses. By using optimized reaction conditions, 1 μg of tissue DNA extracted from nestling budgerigars with histological lesions characteristic of BFDV (lanes marked BFDV) and 1 ng each of HPV-1, HPV-6, bovine papillomavirus type 1 (BPV1), deer papillomavirus (DPV), and SV40 were amplified by PCR. Reaction products were routinely electrophoresed on an ethidium bromide-agarose gel. A *Hin*dIII digest of lambda phage DNA was included as a molecular mass marker (lane L). The sizes, in base pairs (BP), are given adjacent to selected DNA fragments. A relatively intensely staining 550-bp fragment was present in the two BFDV samples. A faint, approximately 1,800-bp fragment was visible in the HPV1 sample. (B) The DNA fragments from the agarose gel were transferred to a nylon membrane and probed with a ^{32}P-labeled BFDV-derived probe. After a 2-h exposure, the probe was seen to hybridize only with the BFDV 550-bp fragment.

papovavirus DNAs (HPV-1, HPV-6, bovine papillomavirus type 1, and SV40). Only the BFDV sample produced a strong 550-bp band (Fig. 2A). Another band of approximately 950 bp was also seen in some BFDV-containing samples, but not in the controls (best visible in Fig. 3B). PCR analysis of the control viral DNAs yielded only a single, approximately 1,800-bp, product from one sample (HPV-1). Southern hybridization of these DNA fragments with the BFDV-derived ^{32}P-labeled probe showed that only the 550-bp product from the BFDV sample hybridized with the probe (Fig. 2B). Consequently, the other visible band must have been a PCR artifact unrelated to the 550-bp BFDV sequence.

Computer analysis of the sequences of the SV40 (simian), JC virus (human), and mouse polyomavirus showed that within the VP1 open reading frame of each virus there are sequences with various degrees of homology to both primers A and B. The highest degree of homology for SV40 was 19 of 24 bp (primer A) and 14 of 24 bp (primer B); that for mouse polyomavirus was 15 of 24 bp (primer A) and 17 of 24 bp (primer B); and that for JC virus was 18 of 24 bp (primer A) and 15 of 24 bp (primer B). Other areas of homology with the primers were also found in each virus outside of the VP1 open reading frame. None of these areas had more than 17 of 24 overlapping base pairs.

Detection of polyomavirus in other avian species by using BFDV primers. To investigate the relationship of BFDV to other suspected avian polyomaviruses, purified DNA from a lovebird viral isolate characterized as a papovavirus and suspected of being a polyomavirus and tissue DNA (spleen and liver) from a Hahn's macaw and a sun conure with

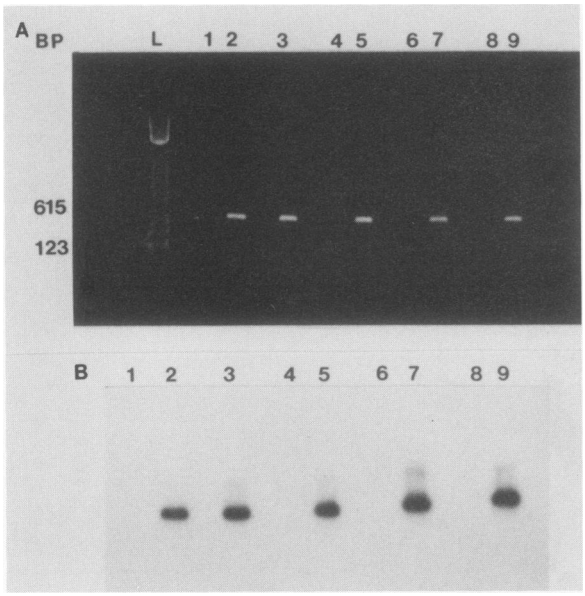

FIG. 3. (A) PCR products of DNA suspected of containing avian polyomavirus and DNA from control birds. Fifteen microliters of each reaction mixture was electrophoresed on an ethidium bromide-agarose gel. Lanes 2, 3, 5, 7, and 9 contain the amplified products from purified DNA samples from CEFs infected with BFDV, pooled organ tissue from a nestling budgerigar, purified viral DNA from a lovebird isolate, pooled spleen and liver DNA from a Hahn's macaw, and pooled spleen and liver DNA from a sun conure with histological lesions suggestive of a polyomavirus infection, respectively. DNA from a chicken embryo (lane 1), DNA from the liver of a peach-faced lovebird dying with psittacine feather and beak disease (lane 4), blood DNA from a healthy yellow-collared macaw (lane 6), and blood DNA from a healthy Patagonian conure (lane 8) are negative controls. The left lane (L) contains DNA base pair markers; the sizes, in base pairs (BP), are marked adjacent to selected DNA fragments. The DNA fragments on this gel were transferred to a nylon membrane and hybridized as described in the legend to Fig. 1. (B) Autoradiogram of the hybridized filter after a 1-h exposure. Numbered lanes correspond to those in panel A.

histological lesions characteristic of polyomavirus infection were amplified by using the optimized reaction conditions. PCR products from all three samples displayed a relatively intense (++++) 550-bp band and a faint 950-bp band, as did the BFDV samples on an ethidium bromide-agarose gel (Fig. 3A). Control DNAs from a chicken, lovebird, macaw, and conure had one to two faint, larger DNA fragments (Fig. 3B). The BFDV-derived probe strongly hybridized with each of the 550-bp products and more weakly with the 950-bp band. The faint variable bands derived from the control samples did not hybridize with the probe (Fig. 3B).

Restriction enzyme mapping of PCR products. To confirm that the 550-bp fragment amplified from the budgerigar sources had the expected sequence and to compare it, on a limited basis, with the sequences of the amplified products originating from the other three species, four restriction enzyme (*Taq*I, *Alu*I, *Bam*HI, and *Pst*I) digests were made of each 550-bp fragment. All four amplification products had identical restriction patterns with each of the four enzymes. A representative digest (*Taq*I) is shown in Fig. 4. The number and location of the restriction cuts were exactly at the sites predicted by the published BFDV sequence (27): *Taq*I (two restriction sites), *Alu*I (1 restriction site), and

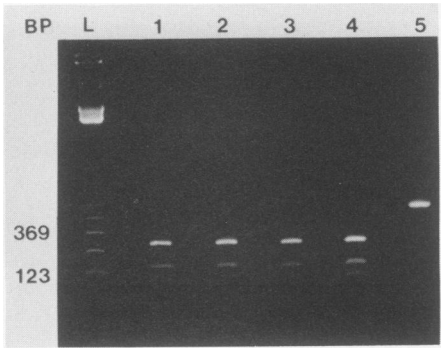

FIG. 4. *Taq*I restriction enzyme digestion of PCR amplification products originating from four parrot species. An ethidium bromide–1.5% agarose gel of PCR products from budgerigar (lane 1), peach-faced lovebird (lane 2), Hahn's macaw (lane 3), and sun conure (lane 4) is shown. Lanes 1 to 4 were digested with *Taq*I endonuclease. Lane 5, Undigested reaction products from a budgerigar sample; lane L, a 123-bp ladder, as in Fig. 3A.

*Bam*HI (one restriction site). Although it cut control DNA under identical conditions, *Pst*I did not cut any of the PCR products at the predicted site.

PCR analyses of tissue DNA and serum from flocks affected with BFDV. Nestling, adult, and embryo tissue DNAs and nestling and adult sera were analyzed by PCR. Representative samples are displayed in Fig. 5. Controls of purified lovebird-derived polyomavirus DNA (20 and 2×10^4 copies) with 1 μg of chicken embryo DNA were amplified and run on the same gels as the clinical samples (data not shown). Positive results (Table 2) were graded on a scale of + to ++++, reflecting the approximate number of copies of the target sequence in the initial reaction mixture. Of the 18 budgerigars whose tissue DNA was analyzed, all 7 nestlings were positive (+++ to ++++). Of the ++++ samples, some were estimated to have had 2×10^6 or more copies in the original reaction mixture (Fig. 1). The results for three adult samples of tissue DNA were ++; and results for the embryo samples were ++ (one sample), + (five samples), and negative (two samples). Because 1 μg of tissue DNA is approximately equivalent to the cellular DNA from 5×10^5 avian cells (7), these results can also be expressed as the number of copies of the target sequence per number of cells.

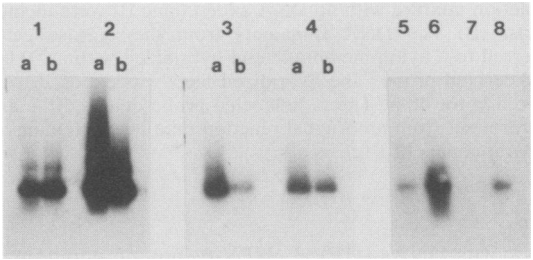

FIG. 5. Autoradiograph of clinical samples hybridized with the BFDV-derived probe. PCR products were electrophoresed, transferred to a nylon membrane, and hybridized with the BFDV-derived ^{32}P-labeled probe. The membrane was exposed to radiographic film for 48 h. Lanes 1a and 2a, 3a and 4a, and 5 to 8 are amplified products from tissue DNA from nestling, adult, and embryo DNAs, respectively. Amplification products from serum samples are shown for each of the nestlings (lanes 1b and 2b) and adult birds (lanes 3b and 4b).

TABLE 2. Results of PCR analysis of adult, nestling, and embryo budgerigar tissue DNAs and adult and nestling sera

Sample	Estimated no. of copies in sample[a]			Histological lesions suggestive of polyomavirus infection
	Tissue DNA[b]	Serum[c]		
		Untreated	Boiled	
Adults				
1	++	—[d]	+	No
2	++	—	+	No
3	++	—	++	No
Nestlings				
1	++++	Negative	Negative	Yes
2	++++	+++	+++	Yes
3	++++	+++	++++	No
4	++++	+++	++++	Yes
5	++++	++	++++	Yes
6	+++	++	++/+++	No
7	+++	Negative	+	No
Embryos				
1	++	—	—	—
2	+	—	—	—
3	+	—	—	—
4	+	—	—	—
5	+	—	—	—
6	+	—	—	—
7	Negative	—	—	—
8	Negative	—	—	—

[a] +, <200 copies; ++, 200 to 2×10^4 copies; +++, 2×10^4 to 2×10^5 copies; ++++, $>2 \times 10^5$ copies.
[b] Copies per 500,000 cells
[c] Copies per 5 μl of serum.
[d] —, Not done.

In these terms, nestlings had between 0.004 and 4 copies per cell, adults had 4×10^{-4} to 4×10^{-5} copies per cell, and embryos had 3×10^{-6} or fewer copies per cell.

PCR analysis was also conducted on serum samples. It was found, using the sera from nestlings, that PCR analysis of boiled sera was more sensitive than PCR analysis of untreated serum (Table 2). Therefore, adult sera were analyzed only after boiling. While the sensitivity of the PCR was not determined specifically for the detection of virus in serum, a wide range of product concentrations were produced that were comparable to those yielded by tissue DNA samples. Therefore, the product yields from the sera were graded on the same scale as the purified DNA samples were. It was realized, however, that the values derived from serum samples may not correlate precisely with the same values derived from tissue DNA samples. Values obtained from nestling sera ranged from negative to ++++, with only four being in the range of +++ to ++++. One nestling had a negative serum result, even though it had a ++++ value for its tissue DNA. All three adult samples were positive, in the range of + to ++. There was no correlation between values for tissue DNA and those for serum or tissue DNA and histological lesions of polyomavirus infection.

PCR analysis of culture fluids and Formalin-fixed tissues. Pooled allantoic fluid from chicken eggs infected with the lovebird viral isolate was positive (visible on an ethidium bromide-agarose gel) without any pretreatment. Untreated tissue culture fluid containing freeze-thawed cells from the lovebird viral isolate and BFDV-infected CEFs were negative, but both became positive (+++) after they were boiled. Uninfected tissue culture fluid and allantoic fluid controls were both negative (data not shown). DNAs derived from the two Formalin-fixed tissue samples were negative.

DISCUSSION

We demonstrated that by using a single set of oligonucleotide primers located within the putative coding region for the VP1 protein of BFDV, PCR can be used to detect the BFDV genome in DNA extracted from budgerigar tissues, sera of some of these same birds, and chicken embryo allantoic fluid and CEF cultures infected with BFDV. The observed 550-bp amplification product was shown to be derived from BFDV DNA by the demonstration of four restriction sites predicted from the published sequence. A *Pst*I site also predicted by its sequence was not present. Other authors (6) also failed to find this *Pst*I site in another BFDV isolate, suggesting genetic variation among different BFDV strains. By using these primers, PCR was extremely sensitive, being able to detect as few as 20 copies of the target sequence in 1 μg of cellular DNA with 40 repetitions of cycle 2. An increase in the repetitions of cycle 2 to more than 40 would increase the sensitivity further at the level of the ethidium bromide-agarose gel. However, it would also increase the amount of nonspecific fragment amplification, making interpretation more difficult; therefore, it was not done. The determination of the sensitivity of PCR for the detection of BFDV in serum, allantoic fluid, and tissue culture fluid was not done in this investigation. However, judging from results of a previous report (18), a sensitivity similar to that reported here for purified DNA samples might be expected, given proper sample preparation. The increased sensitivity observed following boiling of sera and CEF culture fluid could be explained by disruption of the virions, which exposes their nucleic acid, or by the reduced presence of cellular products which are known to interfere with the *Taq* polymerase (29).

PCR amplification with these primers, as well as being sensitive, was highly specific for avian polyomavirus. PCR did not detect DNA from a mammalian polyomavirus (SV40). Comparison of the SV40 sequence with those of JC virus (human) and mouse polyomavirus predicted that binding of primers to these sequences under the selected PCR conditions was also unlikely. While several nonspecific fragments were amplified from the HPV-1 sample and cellular DNA, all of these products were substantially larger than the authentic BFDV 550-bp product. More importantly, none of these products hybridized with the BFDV-derived ^{32}P-labeled probe.

Purified DNA from a suspected polyomavirus isolated from a lovebird and tissue DNA from a Hahn's macaw and a sun conure with histological lesions suggestive of polyomavirus infection were analyzed by PCR. All three samples yielded a 550-bp fragment with the same four identical restriction enzyme sites present in the BFDV-derived fragment, and all were missing the *Pst*I restriction site. Also, under stringent conditions, all three products hybridized with a BFDV-derived radiolabeled probe. This evidence, coupled with the specificity of the probe, shows that the viral agent isolated from the lovebird was a polyomavirus and that polyomavirus genomes were present in diseased tissues of the Hahn's macaw and the sun conure. Although the exact relationship between these viruses and BFDV was not demonstrated precisely by the results of this study, the conservation of restriction sites could be explained either by the fact that they were similar viruses with an area of highly conserved sequences or that they were the same virus. To

define the relationship between these viruses further, the amplified 550-bp product could be sequenced. Additionally, other portions of each virus could be amplified and sequenced by using different sets of primers. Then, comparison of the extents of sequence homology between different amplified regions would confirm or refute the identities of the four viruses.

Identification of the viral genome in the sera of both adult and young budgerigars suggests that PCR could be used as a rapid screening procedure for the detection of BFDV in an aviary where this disease is suspected. Individual birds can also be screened for exposure to the virus. However, it must be noted that one nestling with high tissue levels of virus had no detectable virus in its serum. Potentially, PCR could be used to screen other psittacine birds in the same manner.

The amount of viral genome in nestling, adult, and embryo samples varied over 10^6-fold from group to group. The highest copy numbers detected (at times over 4 copies per cell) were in nestlings. This is best explained by active viral replication. The presence of the viral genome in the sera of these same birds, estimated to be in excess of 2×10^6 copies per 5 μl, suggests that these birds experienced a viremia. Because most of the serum samples had some degree of hemolysis, it is uncertain whether the virus was free or cell associated. High copy numbers either in serum or tissue DNA could not be correlated with disease, because some of these birds had no histological lesions suggestive of polyomavirus-induced disease and appeared to be growing normally.

There is more than one possible explanation for the lower copy numbers found in adult tissue DNA and sera and the copy numbers found at the lowest level of sensitivity in embryos. Copy numbers of 200 to $2 \times 10^4/5 \times 10^5$ cells were found in adult birds. This range could indicate previous exposure to the virus, with integration of all or part of the virus genome into some of the cells. Viral integration is known to occur with other polyomaviruses (33). Alternatively, it may indicate a persistent low-level infection. Viral genomes in the sera of these birds could also originate from a low-level viremia or from integrated virus in DNA from lysed blood cells. Copy numbers in embryo tissue DNA are generally lower than those in adult tissue DNA, with 7 of 8 being fewer than 200 copies per 5×10^5 cells. This copy number is far too low to be explained by passage of integrated virus in the germ line, because at least one copy per cell would be expected. Another possible explanation for the presence of this genome includes in ovo transmission of the whole virus, either by incorporation of the virus into the egg before hatching or penetration through the shell after the egg is laid. This explanation is consistent with a previous report (3), in which virus-induced lesions were present in newly hatched nestlings. The concentration of the viral genome varies little between embryos at different stages of development, suggesting that active viral replication, if it occurs at all, is minimal. While considerable care was taken to prevent environmental contamination of samples, it cannot be entirely ruled out that there was an exogenous origin of the very small copy number seen in the embryos and in some of the adults.

In some situations, PCR can be substituted for virus isolation as a diagnostic tool. Additionally, it can be used to confirm the identities of viruses isolated by more conventional methods. PCR may also prove useful in assays such as virus neutralization, because it could prove to be a more sensitive measure of infectivity than visual observations of cytopathic effect or fluorescent-antibody staining.

Previous reports (14, 26) indicate that DNA from Formalin-fixed tissues can be used for PCR analysis. These reports also show that Formalin degrades DNA and that after fixation periods of 2 weeks, DNA quality is severely reduced, making PCR analysis less satisfactory (26). The two samples of Formalin-fixed tissue analyzed in this study yielded no PCR product. Frozen tissue samples originating from the same two birds were positive (++++). It is strongly suspected that the length of storage of these samples in Formalin (more than 1 year) resulted in severe degradation of the DNA, making PCR analysis impossible.

Finally, the potential epidemiologic implications of this investigation are substantial. Persistent viral infection in adults and the potential for transmission through eggs suggest that control of this disease might require elimination of all the budgerigars on the premise, disinfection, and restocking with birds free of the disease. Additionally, the similarity of BFDV and the other avian polyomaviruses described here raises the question of whether there may be transmission between budgerigars and other parrots.

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